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ADAM-17/FHL2 colocalisation suggests interaction and role of these proteins in colorectal cancer

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Abstract

FHL2 is a multifunctional scaffolding protein; its expression is associated with poor prognosis in colorectal cancer. ADAM-17 is a metalloprotease implicated in ectodomain shedding. FHL2 regulates ADAM-17 plasma membrane localisation, and FHL2 deficiency leads to decreased activity of ADAM-17 in mouse macrophages. Presence and relationship of the ADAM-17/FHL2 complex with colorectal cancer progression is unknown. We studied FHL2 and ADAM-17 expression in several colon cancer cell lines by immunocytochemistry and western blot. To highlight the interaction between both molecules, we used the Duolink® kit for proximity ligation assay on SW480 cells. We also performed proximity ligation assay on biopsies and surgical specimens of colorectal adenocarcinoma and on matched normal mucosa. Furthermore, biopsies of colorectal adenoma with matched normal mucosa were selected. For quantification, pictures of the malignant, adenomatous and normal tissues were taken. Proximity ligation assay signals were quantified. Mean numbers of proximity ligation assay signals and of proximity ligation assay signals/nucleus were calculated. All cell lines showed FHL2 immunoreactivity; strongest positivity was observed in SW480 cells. ADAM-17 was expressed in all cell lines. Proximity ligation assay signals were present in SW480 cells. Quantitative analysis revealed that the interaction between FHL2 and ADAM-17 is more frequent in malignant than in normal tissue ($p = 0.005$). The mean number of ADAM-17/FHL2 proximity ligation assay signals was higher in colorectal adenocarcinoma than in adenoma with low-grade dysplasia ($p = 0.0004$). FHL2 interacts with ADAM-17 in normal, dysplastic and malignant colon epithelial cells. Colocalisation of these proteins is more frequent in malignant than in normal and dysplastic cells, suggesting a role for ADAM-17/FHL2 complex in the development of colorectal cancer.

Keywords

ADAM-17, FHL2, colorectal cancer, proximity ligation assay

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Introduction

Colorectal cancer represents the third most common cancer and the third leading cause of cancer death in men and women in the United States.[1] The prognosis is strongly linked to the stage at the diagnosis: The 5-year survival rate is 90% for localised disease, while this rate falls to 13% in metastatic disease at the time of diagnosis.[2] This high mortality rate is due to acquired resistance of colorectal tumoural cells against conventional chemotherapeutic agents or targeted therapy drugs.[3] Currently, predictive response to targeted therapy drugs, like monoclonal antibodies directed

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against epidermal growth factor receptor (EGFR), is conditioned mainly by mutational status: Anti-EGFR monoclonal antibodies are effective in patients whose tumours do not harbour *KRAS, NRAS, BRAF* and *PIK3CA* mutations.[4]

EGFR ligands are synthesised as transmembrane precursors that can be cleaved by cell surface proteases, in particular members of the ADAM (a desintegrin and metalloprotease) family. ADAMs convert membrane-associated proteins into soluble effectors and, at the same time, rapidly reduce the level of surface expression. ADAM-17/TACE (tumour necrosis factor-α (TNF-α) converting enzyme) is a membrane-bound enzyme that cleaves ligands of EGFR (e.g. transforming growth factor-α (TGF-α) and amphiregulin) as well as other cell surface proteins such as cytokines (e.g. TNF-α), cytokine receptors (e.g. interleukin-6 receptor (IL-6R) and tumour necrosis factor receptor (TNF-R)) and adhesion proteins (e.g. L-selectin and intercellular adhesion molecule-1 (ICAM-1)).[5] ADAM-17 is strongly expressed in colorectal cancer, where it co-expresses with EGFR.[6] Chemotherapy activates ADAM-17 which induces growth factor shedding, growth factor receptor activation and drug resistance.[7] ADAM-17 is an essential component of the EGFR axis, and chemotherapy-induced growth factor shedding via ADAM-17 is promoted by oncogenic *KRAS*.[8] Moreover, the EGFR tyrosine kinase–resistant HCA-7 cell line is growth-inhibited by combined monoclonal antibody therapy and ADAM-17 inhibition.[9] This suggests that ADAM-17 can be a promising therapeutic target and a predictive biomarker in colorectal cancer. ADAM-17 localisation and activity is regulated by the four-and-a-half LIM domain protein 2 (FHL2).[10] FHL2-deficient macrophages have a lower ability to release ADAM-17 substrates, TNFR-1 and TNFR-2, when compared with wild-type macrophages, suggesting a physical and functional interaction between ADAM-17 and FHL2.[10]

We previously showed that FHL2 is overexpressed in colorectal cancer and that its expression is related to overall and disease-free survival.[11] Rather than investigating the mere presence of ADAM-17 and FHL2, we aimed to study the interaction between ADAM-17 and FHL2 in colorectal cancer progression.

Materials and methods

Colon cancer cell lines

Human colorectal cancer cell lines HCT8/E11, SW480 and SW620 were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum (FCS), Fungizone and antibiotics. The HT29 colorectal cancer cell line was grown in RPMI supplemented with 10% FCS (Life Technologies, Gent, Belgium).

Cell lysates and western blots

Human colorectal cell lines were harvested in RIPA lysis buffer. Lysates were suspended in reducing sample buffer (0.5 M Tris–HCl, 40% glycerol, 9.2% sodium dodecyl sulphate (SDS), 4.5% 2-mercaptoethanol and 0.011% bromophenol blue, pH 6.8) and boiled at 95°C for 5 min. Equal protein amounts were separated on 8% (ADAM-17) or 12% (FHL2) polyacrylamide gels, transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), blocked in 5% nonfat milk in phosphate-buffered saline (PBS) with 0.5% Tween-20 (Sigma-Aldrich, Diegem, Belgium) and immunostained. The following antibodies were used: anti-FHL2 (clone 11-134, mouse monoclonal, 1/400; MBL, Woburn, MA, USA) and anti-ADAM-17 (rabbit polyclonal, 1/1000; Cell Signaling, Danvers, MA, USA). Detection was performed by horseradish peroxidase–conjugated secondary antibodies (all GE Healthcare, Pittsburgh, PA, USA) and chemiluminescence (Pierce ECL Western Blotting Substrate; Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Anti-α-tubulin (clone B-5-1-2; Sigma-Aldrich) was used as a loading control.

Cell blocks

Cell culture flasks were washed with 15 mL PBS after medium removing; the cells were stripped using a cell scraper. Cells and PBS were transferred into an Eppendorf for 5 min centrifugation (1000 r/min). Supernatant was removed, and the pellet was suspended into 4% formaldehyde for 20 min at 4°C. This solution was centrifuged again, formaldehyde supernatant was removed and the pellet was suspended into agarose gel. This sample was treated in the Tissue-Tek VIP (Sakura, Alphen aan den Rijn, The Netherlands) and included into paraffin blocks.

Patient samples

We first studied biopsies from 10 patients with colorectal adenocarcinoma and biopsies from 10 other patients with colorectal adenoma with low-grade dysplasia. The validation cohort included surgical specimens from nine patients with colorectal adenocarcinoma. In both cohorts, tumoural and dysplastic samples were matched with normal distant colonic mucosa from the same patients. We also included surgical specimens of metachronous liver metastasis from four patients. Clinical and histopathological characteristics are described in Table 1. All patient samples were included through the opt-out method applied for residual tissue in our hospital.

FHL2 and ADAM-17 immunohistochemistry

Sections of 5 µm were prepared from HCT8/E11, HT29, SW480 and SW620 cell blocks and from paraffin blocks of the first patient cohort. Slides were deparaffinised and pretreated with citrate buffer. Endogenous peroxidase was blocked with normal horse serum.

Slides were incubated for 1 h with primary antibodies at room temperature (FHL2, 30 µg/mL, ab66399, rabbit polyclonal; Abcam, Cambridge, United Kingdom; and

Table 1. Clinical and histopathological characteristics of the studied cases.

	Cohort 1: biopsies		Cohort 2 (validation cohort): surgical specimens
Number of patients	10	10	9
Diagnosis	Adenocarcinoma	Adenoma	Adenocarcinoma
Mean age (minimum–maximum)	64.5 (52–84)	59.6 (36–81)	64.1 (53–80)
Male:female ratio (%)	4:6 (0.67)	4:6 (0.67)	7:2 (3.5)
Anatomic location			
Right colon	3	3	3
Transverse colon	2	0	1
Left colon	4	2	1
Sigmoid	0	2	3
Rectosigmoid	1	1	1
Rectum	0	2	0
Tumoural grade			
Grade I	2		3
Grade II	8		5
Grade III	0		1
Tumoural stage			
T status			
T1			1
T2			3
T3			4
T4			1
N status			
N0			3
N1			6

ADAM-17, 1/50, LS-B4426, mouse monoclonal; Bio-Connect Life Sciences, TE Huissen, The Netherlands). For FHL2 immunostaining, rabbit EnVision+ System-HRP Labelled Polymer (Dako, Heverlee, Belgium) was applied, followed by 3,3′-diaminobenzidine (DAB) substrate–chromogen incubation. For ADAM-17, biotinylated mouse secondary antibody (1/200; Vector Laboratories, Peterborough, United Kingdom) was applied, followed by an incubation with a Vectastain ABC kit (Vector Laboratories), and immunostaining was revealed after incubation with DAB. Sections were counterstained with haematoxylin. Negative controls consisted of replacing the primary antibodies with irrelevant immunoglobulins. ADAM-17 and FHL2 immunohistochemistry were performed on all cell lines. FHL2 immunohistochemistry was realised on the first patient cohort only (biopsies).

Proximity ligation assay

Sections of 5 µm were prepared from paraffin blocks of all patient samples and cell lines. Slides were deparaffinised and pretreated with citrate buffer. Endogenous peroxidase activity was blocked with normal horse serum. Slides were incubated for 1 h with primary antibodies at room temperature (FHL2, 30 µg/mL, ab66399, rabbit polyclonal; Abcam; and ADAM-17, 1/50, LS-B4426, mouse monoclonal; Bio-Connect Life Sciences). After washing with Tris-buffered saline, according to the manufacturer's datasheet, secondary antibodies conjugated with oligonucleotides (Duolink In Situ PLA Probe Anti-Mouse Minus and Duolink In Situ PLA Probe Anti-Rabbit Plus; Sigma-Aldrich) were added and incubated in a pre-heated humidity chamber for 1 h at 37°C. This step was followed by ligation, amplification and, finally, detection (Duolink In Situ Detection Reagents Brightfield; Sigma-Aldrich). Nuclear stain solution was added to the slides for 2 min.

Quantification of proximity ligation assay signals

For the first patient cohort, five pictures (magnification: ×400) were taken from each slide using Olympus BX51 and Olympus cellSens Entry 1.14 software. ADAM-17/FHL2 proximity ligation assay (PLA) signals were counted by a pathologist (L.V.) to determine the mean number of PLA signals/field.

For the validation cohort, 20 pictures (magnification: ×400) were taken from each slide, except for two patients for whom we were limited to either 19 pictures from the tumour or 12 pictures from the normal colonic mucosa. On these pictures of the validation cohort, PLA signals and nuclei were counted using the cell counter application of Fiji software (http://fiji.sc/Fiji). Supplementary Figure 1 illustrates the quantification process. The mean number of

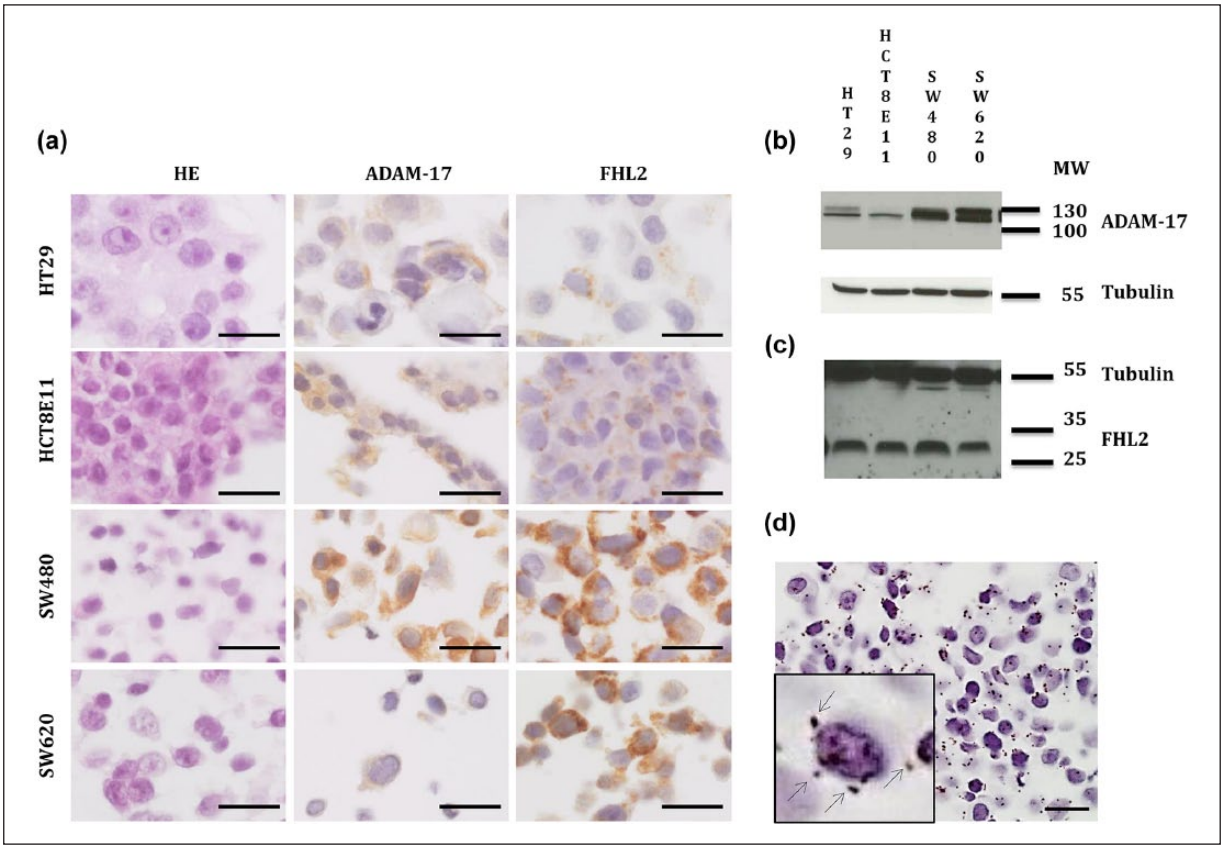

Figure 1. (a) Immunohistochemical expression of ADAM-17 and FHL2 in HT29, HCT8/E11, SW480 and SW620 cells (scale bars: 20 µm). (b) ADAM-17 (upper panel) and tubulin (lower panel) and (c) FHL2 western blotting in HT29, HCT8/E11, SW480 and SW620 cells. (d) ADAM-17/FHL2 PLA in SW480 cells: Brown dots indicate PLA signals (scale bar: 20 µm). Insert: high-power view of a single cell; arrows indicate PLA signals.
MW: molecular weight.

PLA signals/field and mean number of PLA signals/nucleus were calculated for each case. The same quantification process was used for the matched liver metastases.

Statistical analysis

A non-parametric Wilcoxon signed-rank test was used to compare matched groups, while a Mann–Whitney U test was used for non-paired samples. All statistical analyses were carried out using Statistica (StatSoft, Tulsa, OK, USA).

Results

Immunohistochemical study, western blot and ADAM-17/FHL2 PLA on colon cancer cell lines

All human cancer cell lines expressed ADAM-17 and FHL2 at least focally. Immunohistochemical expression of ADAM-17 in the SW620 cell line appeared weak, although western blotting revealed a strong band for this protein; this discrepancy might be related to the sensitivities of the different antibodies that have been used. Regarding immunohistochemical expression and western blotting, the SW480 cell line exhibited the highest level of both proteins (Figure 1(a)–(c)) and, therefore, seemed most suited for PLA. PLA demonstrated ADAM-17/FHL2 colocalisation in SW480 (Figure 1(d)).

FHL2 immunohistochemical expression and ADAM-17/FHL2 PLA in the first cohort (biopsies)

We found higher FHL2 expression in invasive neoplastic cells than in normal and dysplastic adjacent colonic mucosa in which FHL2 expression was generally faint and focal. As we observed previously,[11] neoplastic cells showed cytoplasmic expression with enforced plasma membrane expression (Figure 2), whereas nuclear expression was not detected. Higher expression of FHL2 in colorectal adenocarcinoma compared to normal tissue has also been described by others,[12] but to our knowledge, we are the first to study FHL2 expression in colonic adenoma. Faint FHL2 expression in normal and dysplastic colonic

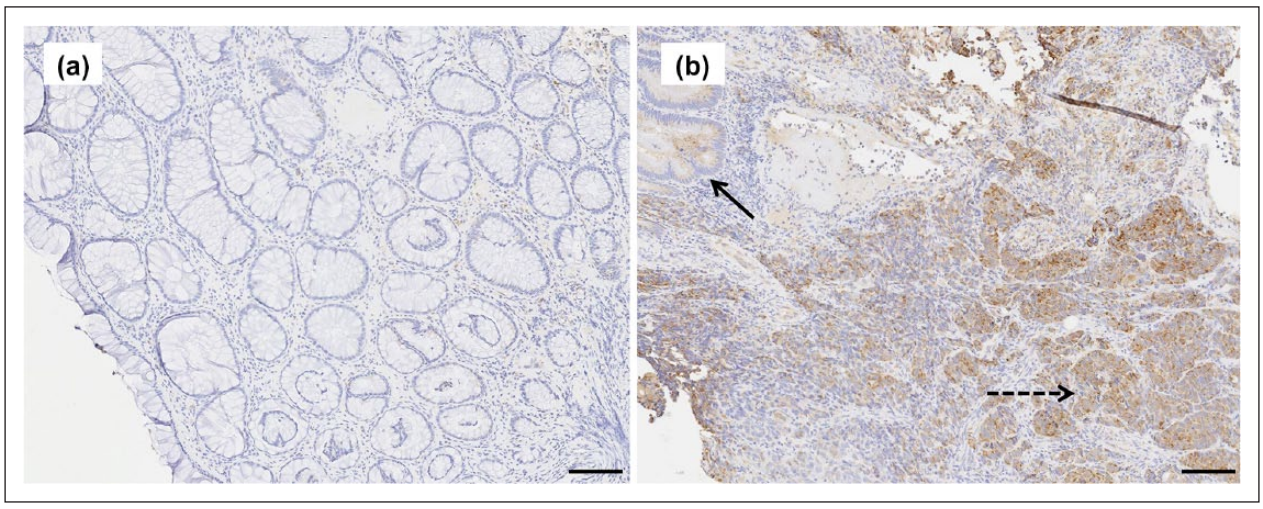

Figure 2. (a) No FHL2 immunohistochemical expression observed in normal colonic mucosa, while (b) increased expression is noted in invasive neoplastic cells (dotted arrow) compared to dysplastic glands (solid arrow; scale bar: 100 µm).

mucosa compared to the elevated FHL2 expression in colonic adenocarcinoma suggests a role of this protein in colorectal carcinogenesis.

ADAM-17/FHL2 PLA revealed a mean number of 110 signals/field in adenoma with low-grade dysplasia compared to 45/field in matched normal tissue ($p=0.047$; Figure 3(a)), and a mean number of 428/field in colorectal adenocarcinoma compared to 71/field in matched normal tissue ($p=0.005$; Figure 3(b)). The mean number of signals/field was higher in colorectal adenocarcinoma than in non-matched adenoma with low-grade dysplasia ($p=0.0004$; Figure 3(c)).

ADAM-17/FHL2 PLA in the validation cohort (surgical specimens)

As also observed in the first cohort, the mean number of ADAM-17/FHL2 PLA signals/field was higher in colorectal adenocarcinoma (395) than in matched normal colonic mucosa (144) ($p=0.01$; Figure 4(a)). Furthermore, the number of ADAM-17/FHL2 PLA signals/nucleus was higher in carcinoma tissue than in normal tissue ($p=0.0077$; Figure 4(b)). Figure 5 illustrates the difference in the number of PLA signals between normal colonic mucosa and colorectal adenocarcinoma. The mean numbers of ADAM-17/FHL2 PLA signals/field and of ADAM-17/FHL2 PLA signals/nucleus were higher in three of the studied liver metastases than in the corresponding primary tumours. One patient presented lower numbers in the metastasis than in the primary tumour (Figure 4(c) and (d)).

Discussion

The FHL2 protein is upregulated in colorectal, breast, prostate and ovarian cancers.[13] High FHL2 expression is related to poor overall and metastasis-free survival in colorectal cancer.[11] This poor prognosis might be related to the role of FHL2 in epithelial to mesenchymal transition (EMT) in which FHL2 regulates E-cadherin transcription through its interaction with Snail1, a potent inducer of EMT.[12] Immunohistochemical study of FHL2, E-cadherin and β-catenin expression on serial slides of colorectal adenocarcinoma revealed that invasive neoplastic cells exhibiting high FHL2 expression with plasma membrane pattern showed a loss of E-cadherin and a nuclear β-catenin expression.[11] This suggests that FHL2 can interact with a plasma membrane partner implicated in the EMT process.

ADAM-17 is a plasma membrane sheddase implicated in EGFR and TNFR transactivation and in interleukin-6 (IL-6) signalling.[14] ADAM-17 is strongly expressed in oesophageal squamous cell carcinoma, and its expression is related to lymph node metastasis and tumour, node, metastasis (TNM) stage.[15,16] In addition, ADAM-17 is related to poor prognosis in gastric[17,18] and in non–small cell lung cancer.[19] ADAM-17 overexpression induces EMT in lung adenocarcinoma cells.[20] So far, there are no data on ADAM-17 and E-cadherin shedding. ADAM-10, the closest relative of ADAM-17,[21] mediates, however, E-cadherin shedding,[22] and ADAM-10 overexpression in epithelial cells increases the β-catenin downstream gene cyclin D1 and enhances cell proliferation.[23]

The ability of PLA to visualise endogenous protein complexes in clinical tissue specimens provides unique opportunities to characterise molecular processes at cellular resolution.[24] In this study, we demonstrated that ADAM-17/FHL2 PLA signals are more frequent in colorectal adenocarcinoma than in adenoma and normal colonic mucosa, suggesting an interaction of these proteins in the final steps of colorectal carcinogenesis. Since we did not include tissues from hyperplastic polyps and serrated adenomas, we

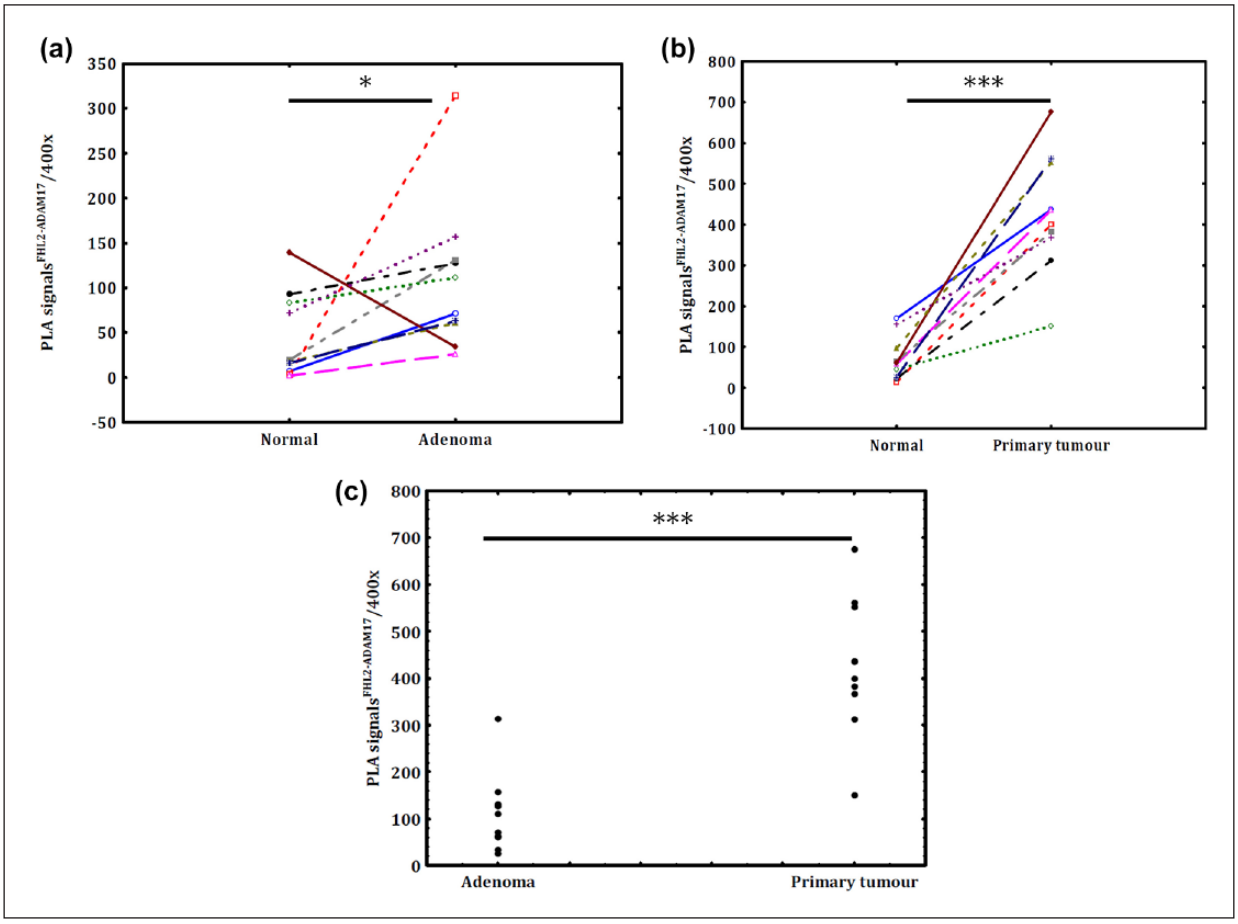

Figure 3. Comparison of the mean number of ADAM-17/FHL2 PLA signals (a) between normal colonic mucosa and matched adenoma with low-grade dysplasia, (b) between normal colonic mucosa and matched colorectal adenocarcinoma and (c) between adenoma with low-grade dysplasia and non-matched colorectal adenocarcinoma.
*$p < 0.05$; ***$p < 0.001$.

can, however, not generalise this finding to the serrated pathway of colorectal cancer. The higher number of signals found in three of the four studied liver metastases compared to the primary tumours might also point to a role for ADAM-17/FHL2 interaction in metastasis development. The detected proximity between the two proteins is not proof of direct physical interactions, but it provides information that both molecules participate in the same protein complexes and thus contribute to the actions of these complexes.[24] Canault et al.[10] also showed that FHL2 interacts with ADAM-17. Under phorbol 12-myristate 13-acetate (PMA) stimulation, FHL2-deficient macrophages had a lower ability to release ADAM-17 substrates, TNFR-1 and TNFR-2, when compared with wild-type macrophages, suggesting a physical and functional interaction between ADAM-17 and FHL2 that implies that FHL2 has a role in the regulation of ADAM-17.[10]

Associating a function to the interaction between proteins is, however, a challenging task that becomes even more complex when each protein possesses several potential functions. The number of identified ADAM-17 substrates is increasing, and evidence is mounting that ADAM-17 has a role in almost every cellular function.[21] Because of the ubiquitous nature of the enzyme, mechanisms for controlling ADAM-17 activity are worth investigating. Our findings open new perspectives with this regard.

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Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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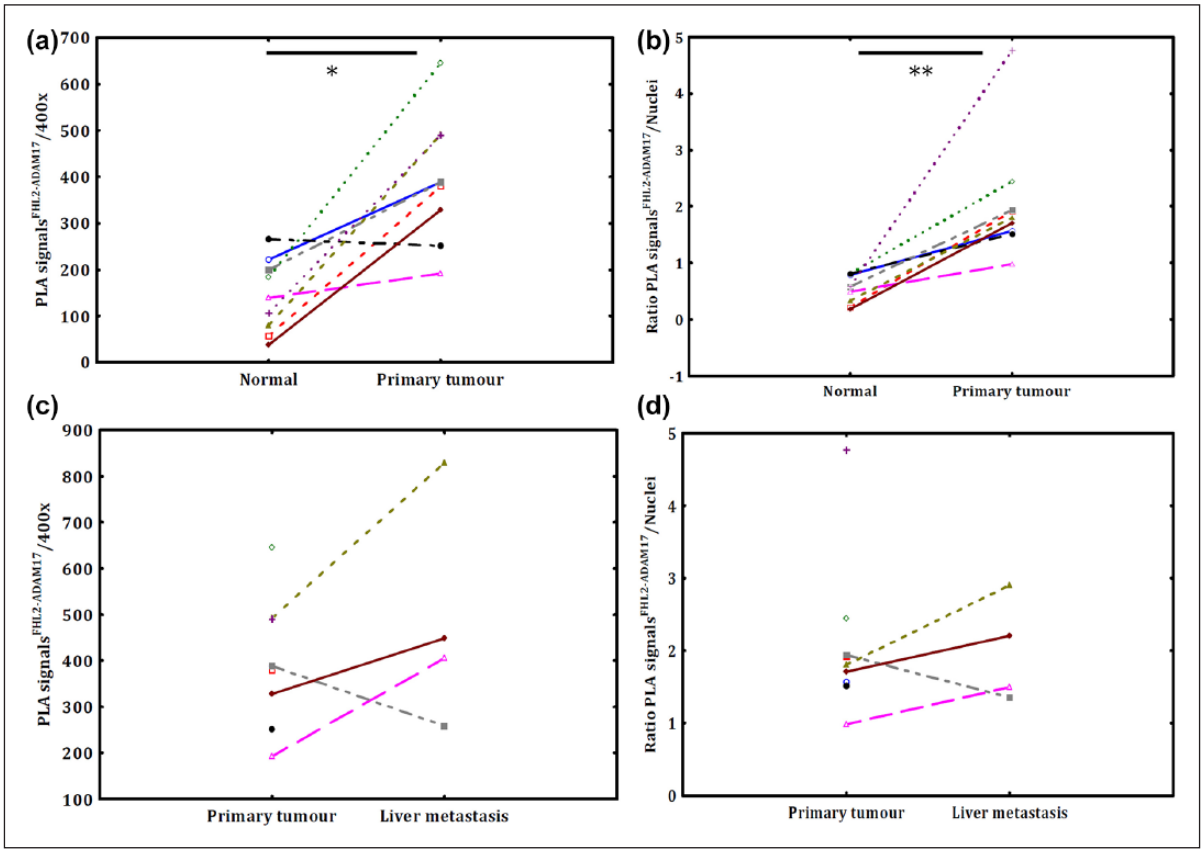

Figure 4. (a) Comparison of the mean number of ADAM-17/FHL2 PLA signals between normal colonic mucosa and matched colorectal adenocarcinoma, (b) comparison of the mean number of ADAM-17/FHL2 PLA signals/nucleus between normal colonic mucosa and matched colorectal adenocarcinoma, (c) comparison of the mean number of ADAM-17/FHL2 PLA signals between colorectal adenocarcinoma and matched liver metastasis and (d) comparison of the mean number of ADAM-17/FHL2 PLA signals/nucleus between colorectal adenocarcinoma and matched liver metastasis.
*$p < 0.05$; **$p < 0.01$.

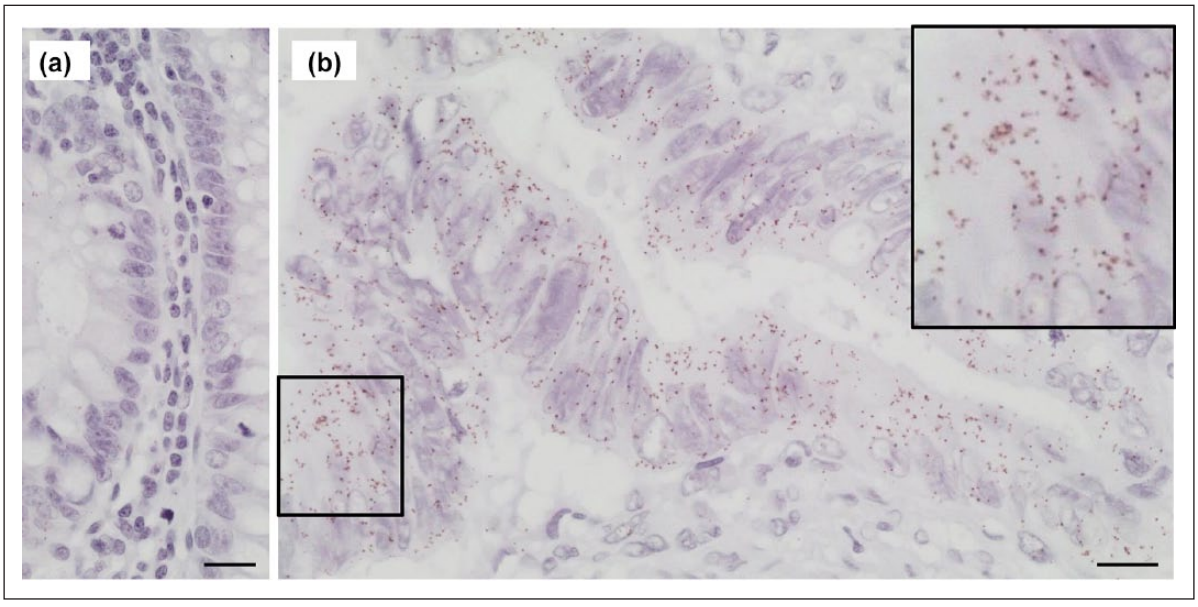

Figure 5. ADAM-17/FHL2 PLA in (a) normal colonic mucosa and (b) colorectal adenocarcinoma (scale bars: 20 µm). Insert right upper corner: high-power view of insert left lower corner.

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